Specific Adoptive Immunotherapy by Local Transfer of Cultured Syngeneic Spleen Cells for Mammary Carcinoma in Mice

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Specific Adoptive Immunotherapy by Local Transfer of Cultured Syngeneic Spleen Cells for Mammary Carcinoma in Mice

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Abstract

Weak in vivo tumor neutralizing activities to syngeneic tumor Shionogi Carcinoma (SC) 42 were observed in the spleen cells of the tumor bearing dd Shionogi (DS) mice on the 14th day after inoculation only when effector to target cell ratio (E/T ratio) was high (E:T=100:1). To remove plastic adherent cells from these spleen cells slightly added to these activities. When the plastic nonadherent splleen cells of the tumor bearing mice were cultured with Mitomycin C (MMC) treated SC 42 tumor cells for 5 days their tumor neutralizing activities were further increased and observed even at low E/T ratio (E : T=10 : 1). Moreover, the spleen cells of the mice in any tumor bearing stage could acquire these antiitumor activities by mixed lymphocyte tumor cell culture (MLTC).

These cultured spleen cells whose anti-tumor activities were augmented by secondary MLTC for 3 days, were proliferated by addition of T cell growth factor (TCGF). The local adoptive transfer of these proliferated cells significantly suppressed the growth of the tumor which had been implanted 5 days before, while the intravenous transfer of these cells was ineffective. When ^{51}Cr labelled syngeneic lymphoid cells proliferated by TCGF were injected into the footpad, the labelled cells stayed there for more than 24 hours. On the other hand, the cells injected intravenously accumulated in the lung first and redistributed to the liver thereafter. It is supposed with these results that the locally transferred spleen cells which had been proliferated by TCGF stayed in the tumor and suppressed the tumor growth.

Introduction

It is generally considered that the lymphocytes of tumor bearing hosts have little anti-tumor activity against autologous tumors. Moreover, it is suggested that these activities are suppressed

Key words: Immunotherapy, T cell growth factor, Interleukin-2, Transfer of lymphocytes, Mouse mammary carcinoma.
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by several humoral[6,8] or cellular mechanisms[2,3]. There are two principal problems for specific anti-tumor immunotherapy. One is how to obtain strong anti-tumor activities, and the other is how to counteract these suppressive mechanisms.

The discovery of T cell growth factor enabled lymphocytes to be proliferated[4,14] and their anti-tumor activities to be augmented in mixed lymphocyte tumor cell culture in vitro[16]. Many experimental models of adoptive immunotherapy with autologous or syngeneic lymphocytes have been reported[1,9,17], but intravenous administration of lymphocytes sensitized in MLTC had only marginal effects[9,17].

In the present study, the spleen cells of tumor bearing mice which had weak anti-tumor activities were augmented in MLTC and proliferated by TCGF. They showed strong tumor neutralizing activities in Winn assay. In adoptive transfer, they significantly suppressed the syngeneic tumor growth when transferred locally, while intravenous injection of these cells was ineffective.

Materials and Methods

Animals.

Inbred 8 to 12 week old male DS mice, sublined from dd mice by brother-sister mating at 1954 by Yamaguchi et al.[21], were obtained from Aburabi Laboratory of Shionogi Pharm. Co., Japan.

Tumors.

Shionogi Carcinoma 42 and Shionogi Carcinoma 115, both of which had been established from spontaneous mammary carcinomas in DS mice[12] were kindly donated by Dr. Yamaguchi and maintained serially in DS male mice by subcutaneous inoculation. Single tumor cell suspension was prepared by treatment of 0.25% trypsin (Difco). When 1×10^5 of SC 42 cells were inoculated subcutaneously or 4×10^5 into the footpad, tumors became palpable in 10 to 17 days after the inoculation. The mice which received no treatment died in 25 to 60 days after the inoculation. Before MLTC, SC 42 cells were pre-incubated for 30 min at 37°C in RPMI 1640 with 50 μg/ml of Mitomycin C

Treatment of spleen cells.

Mice were sacrificed by cervical dislocation, and spleens of 3 to 10 mice in respective group were removed and pooled. Single cell suspensions were obtained by passing through #100 stainless steel mesh. Erythrocytes were lysed for 5 minutes by 0.83% NH_4Cl Spleen cells were washed with Hanks' balanced salt solution and suspended in an appropriate medium.

Whole spleen cells suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) were incubated for 40 min at 37°C in plastic dish (Corning, N.Y.). Plastic nonadherent cells were collected after vigorous agitation.

Culture of spleen cells.

Culture medium for MLTC was RPMI 1640 supplemented with 7% heat inactivated human AB serum (Flow, Virginia), 50 μM 2 Mercaptoethanol, 20 mM N-2hydroxyethylpiperazine-

N'-2-ethane sulfonic acid (HEPES) and 100 μg/ml gentamicin. Plastic nonadherent cells were cultured with the MMC treated tumor cells at alternative responder cell stimulator cell ratio (R/S ratio), i.e. 10 or 50, in Linbro 2 ml well (Flow) at 37°C in 5% CO_2 in air. In the primary MLTC, the spleen cells were adjusted to 5×10^6/ml and cultured for 5 days. In the secondary MLTC, the cells were adjusted to 1.5×10^6/ml and cultured for 3 days.

TCGF and proliferation of lymphocytes.

According to the Gillis' method[5], 1×10^6/ml of spleen cells of Charles-River SD (CD) rats were cultured with 5 μg/ml of Concanavalin A (Miles-Yeda, Israel) n RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 50 μM 2 ME and 20 mM HEPES for 48 hours. Then the culture supernatant was collected and stored at −20°C until use. In cell proliferation, TCGF was added to 50 vol% of medium. Spleen cells were adjusted to 5×10^4/ml and cultured in the medium. In these experiments cells were proliferated fivefold to tenfold in 4 days.

Evaluation of in vivo anti-tumor activity.

Winn's tumor neutralization assay[20] was used to evaluate in vivo anti-tumor activities of spleen cells. Tumor cells and effector cells (cultured or non-cultured spleen cells) were incubated for 20 min before injection. Effector to target cell ratio (E/T ratio) was 10 to 100. The mixtures of cells containing 1×10^5 of tumor cells were injected into the flank of normal DS mice (5 mice for each group) subcutaneously. The mean diameters of tumors were measured every week. Statistical significance was analysed by Student's t test.

In vivo kinetics of cells.

Normal spleen cells and the syngeneic lymphoid cells which were proliferated by TCGF for 6 months were used in this assay. Ten million of these cells were labelled with 500 μCi of ^{51}Cr for 60 minutes and 2×10^6/0.5 ml of these cells were injected to normal DS mice intravenously and 4×10^6/0.1 ml in right footpads of normal mice. These mice were sacrificed at 2 or 24 hours after the injection. The gamma-emmission of various organs were counted in Autowell Aloka (Aloka, Japan).

Results

In vivo tumor neutralizing effects of spleen cells of tumor bearing mice and tumor removed mice.

The spleen cells of the tumor bearing mice on day 14 after tumor cell inoculation had weak anti-tumor activities at high E/T ratio (E : T=100 : 1). On the contrary, they enhanced tumor growth at low E/T ratio (E : T=10 : 1) compared with normal spleen cells. Plastic non-adherent cells of the tumor bearing spleen cells showed stronger anti-tumor activities at high E/T ratio than the whole spleen cells, and enhancement of tumor growth at low E/T ratio was not observed in them (Table 1).

Tumor removed mice rejected rechallenged syngeneic tumor cells. Their spleen cells had remarkable anti-tumor activities at high E/T ratio. Their plastic nonadherent cells completely suppressed tumor growth at high E/T ratio, while no effects on tumor growth were observed at low E/T ratio (Table 1). Enhancement of tumor growth at low E/T ratio was not observed in

Table 1. Winn assay of the spleen cells of t mor bearing and tumor removed mice

Effector Cells[a]	E/T ratio	Tumor size[b]		
		day 14	day 21	day 28
no	–	5.2±3.9[c]	12.5± 7.3	21.3± 6.5
normal whole	100 : 1	2.4±0.4	13.7± 2.1	24.0± 4.6
tumor bearing whole	10 : 1[d]	9.5±3.7	21.1± 2.5	–
	100 : 1	1.4±2.8	4.6± 6.6	11.5±13.1
tumor bearing nonadherent	10 : 1	2.3±2.1	12.1± 6.8	21.6± 8.0
	100 : 1[d]	0	2.6± 4.3	9.7±12.7
tumor removed whole	10 : 1	7.0±6.1	14.5± 5.9	28.1± 7.0
	100 : 1[d]	0	0.7± 1.0	–
tumor removed nonadherent	10 : 1	5.1±5.9	10.7±10.6	20.6±16.5
	100 : 1[d]	0	0	0

a: Whole spleen cells (whole) or plastic nonadherent cells (nonadherent) were effector cells. Tumor bearing mice were mice on day 14 after tumor cell inoculation of 4×10^5 of SC 42 tumor cells into the right footpad. Tumor removed mice were mice on day 28 after tumor cell inoculation which received removal of tumors on day 14. b: Tumor size was measured on certain day after transplantation of mixture of spleen cells and tumor cells. c: Mean tumor diameter (mm)±S.D. d: $p<0.05$ VS "no effector cell" group.

normal mice, either.

Anti-tumor activities of spleen cells cultured with tumor cells.

The plastic nonadherent spleen cells of the tumor bearing mice on day 14 were cultured with MMC treated SC 42 cells at R/S ratio 10 1 or 50 : 1 for 5 days. These cultured spleen cells suppressed tumor growth at low E/T ratio in Winn assay, compared with the spleen cells cultured without the tumor cells. These anti-tumor activities were more intensive in the spleen cells cultured with the tumor cells at R/S ratio 10 1 than at R/S ratio 50 : 1 (Chart 1). Moreover, [^{3}H]thymidine uptake of these spleen cells in MLTC showed the maximum counts at R/S ratio 10 : 1 (Data was not shown).

Anti-tumor activities of spleen cells of mice in various tumor bearing stages were also assessed with Winn assay. In any tumor bearing stages, plastic nonadherent cells cultured with the tumor cells showed more intensive anti-tumor activities than normal cultured spleen cells. The most intensive activities were observed in the plastic nonadherent cells on day 14 after tumor cell inoculation, while the whole spleen cells on day 14 showed only similar anti-tumor activities as normal plastic nonadherent cells (Table 2).

In addition, these spleen cells possessing anti-tumor activities to SC 42 tumor cells did not show any effects on another syngeneic mammary carcinoma SC 115, suggesting tumor specificity (Table 3).

Local adoptive transfer of cultured spleen cells.

The plastic nonadherent spleen cells of tumor bearing mice on day 14 were cultured with the tumor cells for 5 days, which had most intensive antitumor activities as described above. Then, 1×10^7 of these cells were transferred to the footpad of the mouse where the tumor cells

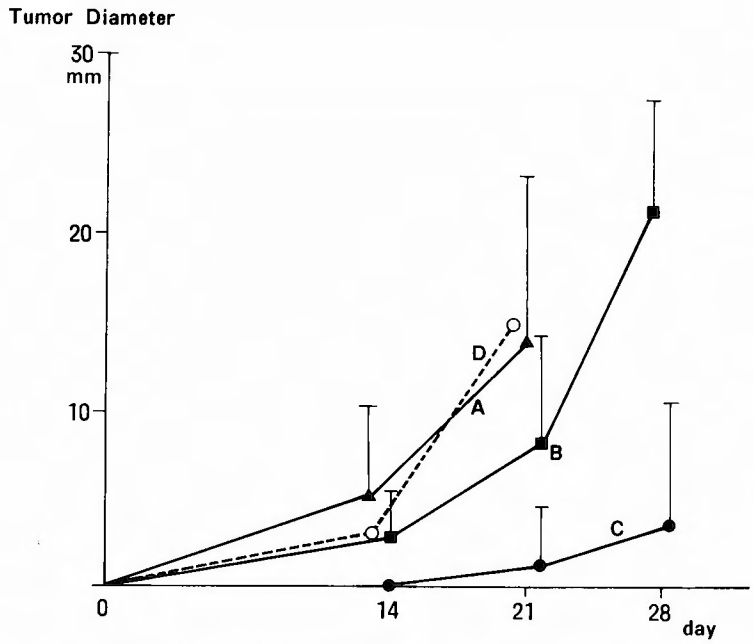

Chart. 1. Anti-tumor activities of cultured plastic nonadherent spleen cells of tumor bearing mice (Winn assay). MLTC was performed by mixed culture of 5×10^6/ml plastic nonadherent spleen cells of 14 days tumor bearing mice with no stimulator (A), 1×10^5/ml MMC-treated SC 42 tumor cells (R/S ratio 50) (B) or 5×10^5/ml MMC-treated SC 42 tumor cells (R/S ratio 10) (C). Plastic nonadherent spleen cells of normal mice were used as control (D). E/T ratio in Winn assay was 10. There were statistically significant differences in tumor growth at i.e. C vs A, B: $p<0.05$ and C vs D: $p<0.01$.

had been implanted 5 days before. This local adoptive transfer of the cultured spleen cells tended to suppress the tumor growth, although the difference between the local adoptive transfer and no treatment was not statistically significant (Chart 2). When these cells were transferred on day

Table 2. Anti-tumor activities of cultured spleen cells of mice in various tumor stages[a]

Effector cells[b]	Tumor weight[c]	Reject/Total
no	7.41±4.03[d]	0/5[e]
normal nonadherent	0.95±1.22	2/4
d. 7 tumor bearing nonadherent	0.46±0.52	2/5
d. 14 tumor bearing whole	1.10±2.02	3/5
d. 14 tumor bearing nonadherent	0.17±0.30	3/5
d. 28 tumor bearing nonadherent	0.66±1.19	2/5

a: Effector cells after 5 days MLTC (R/S ratio 10) were mixed with the tumor cells at E/T ratio 30:1 and transplanted to normal recipient mice. b: effector cells as follows, as control tumor cells only, plastic nonadherent spleen cells of normal mice, plastic nonadherent spleen cells of tumor bearing mice on day 7 after tumor cell inoculation, plastic nonadherent or whole spleen cells of tumor bearing mice on day 14 and plastic nonadherent spleen cells of tumor bearing mice on day 28. c: All of mice were sacrificed 29 days after and tumor weight was measured. d: Mean tumor weight (gm) ±S.D. e: Number of mice rejecting tumor/number of mice tested.

Table 3. The specificity of anti-tumor activities of cultured spleen cells[a]

Effector cell	Target cell	
	SC 42	SC 115
normal spleen cell	9.57±6.39[bd]	2.03±1.85
cultured spleen cell[c]	1.66±0.93[d]	2.92±1.59

a: Anti-tumor activities of cultured spleen cells against different tumor cells were measured with Winn assay (E/T ratio 10) compared with normal spleen cells. b: Tumorweight on day 35. Mean tumor weight (gm) ±S.D. c: Plastic nonadherent spleen cells of SC 42 tumor bearing mice which had been cultured with SC 42 tumor cells for 5 days. d: $p<0.05$.

14 after tumor cell inoculation, suppression of tumor growth was not observed (Data was not shown).

To augment therapeutic effect of the adoptive transfer, the schedule of the transfer was planned as follows. Firstly 7×10^6 cells from primary MLTC were transferred intravenously or locally to the footpads where the tumor cells had been implanted 5 days before. The remaining cells of primary MLTC were cultured with the tumor cells for 3 days (secondary MLTC) and then proliferated with TCGF for 4 days. By this culture with TCGF the cells increased about fivefold to tenfold. This culture was repeated three times and 1.2×10^6, 1.4×10^6 and 1.7×10^6 of the cells were transferred in 4 days interval, respectively. In 5 of 9 mice with this local adoptive transfer the tumor growth was completely suppressed, while only one of 9 mice became tumor free with the local transfer of normal spleen cells (Chart 3). Intravenous transfer of these proliferated cells had no effects on tumor growth.

In vivo kinetics of the spleen cells proliferated by TCGF.

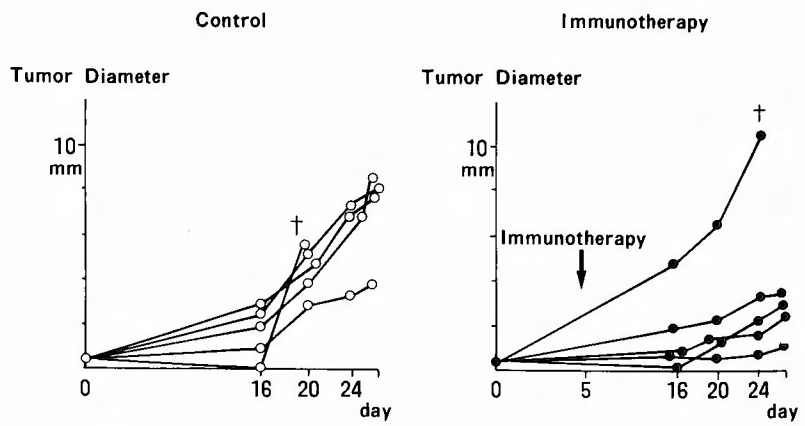

Chart. 2. Effect of local adoptive transfer of cultured spleen cells. After 5 day MLTC (R/S ratio 10:1), 1×10^7 of the plastic nonadherent spleen cells of tumor bearing mice on day 14 were transferred to the footpads where 4×10^5 of SC 42 tumor cells had been implanted 5 days before. The tumor growth was compared with mice which received no transfer. Tumor diameter=the thickness of footpad inoculated—the thickness of the opposite footpad.
†: mouse which died because of tumor growth.

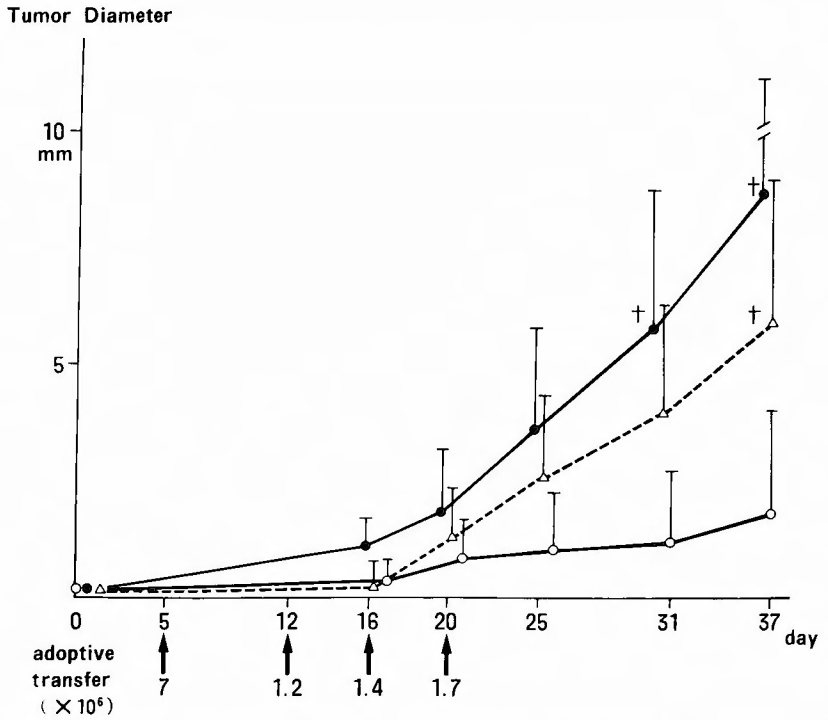

Chart. 3. Effect of adoptive transfer of TCGF proliferated spleen cells. On day O MLTC was started using 5×10^6/ml plastic nonadherent spleen cells of 14 days tumor bearing mice mixed with 5×10^5/ml MMC-treated SC 42 tumor cells. On day 5 spleen cells after MLTC were transferred into the tail vein or to the right footpad where 5×10^5 of SC 42 tumor cells had been inoculated on day 0. Recipient mice were randomized on day 5. Spleen cell culture were followed by secondary MLTC at R/S ratio 10 and then spleen cells were proliferated in TCGF. The proliferated spleen cells were repeatedly transferred intravenously (●) or into the same footpad (○) on day 12, 16 and 20. The same number of spleen cells of normal mice were transferred into the right footpad (△). The recipient mice used were consisted of 8, 9, 10 mice for each group. Among them, (●), 0, (○) 5, (△) 1 mice survived tumor free. Tumor growth were different significantly between (△) and (○). $p<0.001$.

The spleen cells became large blast cells when they were proliferated by TCGF, and their in vivo kinetics would be much different from those of normal cells. Moreover, difference of the cell distribution between the routes of transfer, i.e. local and intravenous, must be one of the most important problems in their effectiveness on tumor growth.

In order to detect in vivo kinetics of the transferred cells, lymphoid cells proliferated by TCGF for 6 months and normal spleen cells were labelled with ^{51}Cr and transferred either intravenously or locally to the foodpad of normal mice. TCGF proliferated cells injected intravenously were accumulated mainly to the lung first and redistributed mainly to the liver, while normal spleen cells were rapidly accumulated to the liver and the spleen and remained thereafter. On the other hand, both TCGF proliferated cells and normal spleen cells which were injected in footpads stayed at the injected site over 24 hours after injection (Table 4).

Table 4. RI uptake of various organs and tissues after ^{51}Cr labelled cells injection[a]

Organ or tissue	Normal spleen cells[a]		TCGF proliferated cells[b]	
	2 hours[c]	24 hours[c]	2 hours	24 hours
Local injection (right footpad)				
right footpad	91.8[d]	31.8	81.3	45.1
left footpad	0.5	0.5	0.8	0.8
right inguinal lymph nodes	0.3	0.3	0.2	0.2
left inguinal lymph nodes	0.3	0.3	0.2	0.2
intestine	1.4	2.1	3.8	3.6
lung	0.4	0.3	0.6	0.4
liver	2.0	2.6	4.9	6.9
spleen	0.3	0.3	0.4	0.4
kidney	3.0	3.4	7.9	7.5
total	100		100	
Intravenous injection				
right footpad	0.8	0.9	0.4	0.3
left footpad	0.8	0.9	0.4	0.3
right inguinal lymph nodes	0.5	0.5	0.2	0.1
left inguinal lymph nodes	0.5	0.4	0.2	0.1
intestine	4.4	4.7	1.1	1.3
lung	22.2	1.4	67.7	0.9
liver	38.9	23.5	25.3	53.3
spleen	26.2	30.5	1.3	3.2
kidney	5.7	2.9	3.4	3.4
total	100		100	

a: Lymphoid cells grown in TCGF and normal spleen cells were labelled with ^{51}Cr and injected into normal DS mice as described in Materials and Methods. b: Lymphoid cells in SC 42 tumor mass were cultured serially and proliferated with TCGF c: Two hours or 24 hours after injection organs and tissues were collected and their RI activity was measured. d: % of the sum of the mean cpm of several organs at 2 hrs after the injection. Each group consisted of 3 mice.

Discussion

In this study, whole spleen cells of tumor bearing mice had weak antitumor activities at high E/T ratio, but enhanced tumor growth at low E/T ratio. Similar results in Winn assay were reported by Prehn et al.[15]. Trainin et al clarified the tumor enhancing effect of 3LL-tumor bearing spleen cells[18]. They reported the tumor enhancing effect was diminished by the treatment of the cells with anti θ+complement. The tumor enhancing effect of the tumor bearing spleen cells in this experiment was diminished by the removal of plastic adherent cells. Which type of cells has the tumor enhancing effect must be clarified by further experiments. The removal of plastic adherent cells increased anti-tumor activities at high E/T ratio. Specific anti-tumor immunity measured with macrophage migration inhibition test showed similar results in the same tumor-host system[7].

Although the plastic nonadherent spleen cells showed more intensive anti-tumor activities at high E/T ratio, their anti-tumor activities were too weak to suppress the growth of the growing tumors. In the specific adoptive immunotherapy, transferred effector cells should act effectively even in low E/T ratio. Normal spleen cells can acquire the anti-tumor activity by mixed-culture

in vitro with Mitomycin C treated or irradiated syngeneic tumor cells[1,13]. Likewise, spleen cells of tumor bearing host can also be resensitized in vitro[13,11]. In the present study, the plastic nonadherent cells of the tumor bearing mice showed the considerably strong anti-tumor activities after they were cultured with the Mitomycin C treated syngeneic tumor cells, and the spleen cells in any tumor bearing stage obtained more intensive anti-tumor activities than normal spleen cells. These results suggest that spleen cells of tumor bearing mice in any stage can be the source of transferred cells in adoptive immunotherapy. Some authors reported the experimental models of immunotherapy in which in vitro sensitized spleen cells were transferred intravenously to the syngeneic tumor bearing mice. Burton reported that intravenous transfer of cultured lymphocytes was ineffective at 100 of E/T ratio[1]. Treves described the inhibiting effect of lung metastasis in combination with surgical removal of tumor[17]. The favorable therapeutic effect by their intravenous transfer was also reported in combination with chemotherapy by KEDAR[10]. However, these effects were marginal. There may be two problems in specific immunotherapy with adoptive transfer. One is the quality and the quantity of transfered cells, and the other is the route of administration.

We recently found that in another tumor-host system (Meth-A- BALB/c) no effector cells were induced by single MLTC and that repeated MLTC could bring the spleen cells to have tumor neutralizing activities very effectively. In this SC 42—DS system secondary MLTC was done to augment the anti-tumor activities.

During these MLTC, number of the cells decreased. It is necessary either to prepare large number of cells before MLTC or to make the cells proliferate after MLTC for obtaining a sufficient number of transferred cells. T cell growth factor (TCGF) was discovered by GALLO et al.[14] from the supernatant of lymphocytes cultured with PHA. TCGF can not only make T cells proliferate preserving their cytotoxic[4] or helper[19] activities but also enhance in vitro sensitization when used free from lectins[16]. In our immunotherapy model, the cultured cells were incubated with TCGF and they proliferated from 5×10^4/ml to 2 to 4×10^5/ml in 4 days possessing anti-tumor activities. The proliferated cells were transfered locally or intravenously and a small aliquot was remained to proliferate again. This procedure was repeated four times and the proliferated cells were transferred each time. By this procedure the adoptive transfer with sufficient number of the cells can be performed several times even if the source of the cells is small.

MILLIS tried the local transfer of lymphocytes cultured with TCGF and reported that life prolonging effect was observed by transfer of sensitized lymphocytes within 24 hours after tumor implantation[11]. In our experiment, local or intravenous transfer of the proliferated spleen cells was started on day 5 after tumor cell implantation and repeated 4 times with 4 to 8 day interval. In the local transfer group tumor growth was significantly suppressed. On the contrary, intravenous transfer resulted in failure. Moreover, local transfer had no effects on the tumors on day 14 after implantation. These results suggested that this specific adoptive immunotherapy was effective only when tumors were small although settled locally.

It is necessary for the transferred cells to contact with the tumor cells to exhibit their anti-tumor activities. Theoretically locally transferred cells have much more chance to contact with

the tumor cells than intravenously transferred cells. Ineffectiveness in intravenous transfer may also be due to the difference of in vivo kinetics between normal lymphocytes and cultured ones. Lotze reported that cultured blasts were trapped by the lung and then accumulated to the liver[10]. They may be unable to migrate to the peripheral circulation. In vivo kinetics of the transfered cells were studied with ^{51}Cr labelled cells. While normal spleen cells intravenously transferred were rapidly accumulated in the liver and the spleen and remained thereafter, TCGF proliferated cells were trapped by the lung first and then redistributed to the liver. Both TCGF proliferated cells and normal spleen cells stayed in injected sites for more than 24 hours when injected locally. From these results it is suspected that the locally transferred cells stay in the site where they contact with the tumor cells and act on them. Referring to clinical trial, Lotze attempted to transfer the autologous lymphocytes cultured with TCGF intravenously in 3 patients of sarcomas and reported that neither side effects nor therapeutic effects were observed[10]. The invalidity of their adoptive immunotherapy would be ascribed to the route of administration. This type of immunotherapy will be able to abe applied most effectively to the locally advanced cancer in combination with surgical resection. We have already tried to transfer locally the cultured lymphocytes of cancer patients which were re-sensitized with soluble antigen of autochthonous cancer cells and proliferated in TCGF. The report of the clinical effects of the therapy is now in preparation.

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和文抄録

マウス乳癌に対する同系培養リンパ球移入による特異的免疫療法

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菅　典道，大垣　和久，稲本　俊，山崎　信保
仁尾　義則，日笠　頼則

DS 雄マウス及び同系移植乳癌 SC42 を用いて担癌リンパ球を TCGF 下に培養増殖させ再び担癌生体に移入した場合の治療効果を検討した．移殖第2週のプラスチック非附着担癌脾細胞は弱い抗腫瘍能を有するが in vitro で腫瘍細胞を混合培養することによりその抗腫瘍能を強化させることができ，更に TCGF で増殖せしめ得た．培養後の抗腫瘍リンパ球を足蹠移植後5日目の担癌局所にくり返し注入すると有意に腫瘍増殖を抑制し，一方，同じリンパ球を静脈内投与した場合には無効であった．TCGF により長期培養した ^{51}Cr ラベルリンパ球を用いて投与後の生体内動態を観察した場合，静注投与時はすみやかに肺及び肝に集積するが局注投与時には一定時間内は投与局所に止まる傾向が観察された．